



Development of a Rapid, Inexpensive Bioassay for Screening Contaminant Bioavailability in Sediment Using mRNA Profiling

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PURPOSE: This technical note describes how the stress responses of common bioassay organisms can be used to identify toxic contaminants and their bioavailability in sediment. The freshwater non-biting midge, *Chironomus tentans*, responds to contaminant exposure by making proteins to detoxify chemicals or repair damage, in addition to other responses. Midges make new proteins by transcription, or copying, of genes into messenger RNA (mRNA) that are used to make many copies of a protein. A snapshot of what the midge is sensing and how it is responding can be found by looking at the mRNAs that are being made (mRNA profiling). Short exposures to common sediment contaminants result in patterns of mRNA profiles that indicate the class and type of the bioavailable chemical. Short analysis time and very high sample throughput enable this approach to be used as a means to rapidly screen large numbers of sediment samples for exposure and/or toxicity. These analyses are faster and cheaper than traditional priority pollutant analysis with the added potential of identifying bioavailable contaminants by the effect they cause in a bioassay organism.

BACKGROUND: Decisions on the identification, assessment, and management of contaminated dredged material are frequently limited by information on how much risk the material poses. A tiered assessment framework has been adopted to determine the suitability of dredged material for disposal (U.S. Environmental Protection Agency/U.S. Army Corps of Engineers (USEPA/USACE) 1991). According to this framework, if there is reason to believe contamination, sediment samples should be collected, extracted, and analyzed. Chemical analyses are developed to maximize recovery of pre-targeted contaminants of interest. However, a manager must know what chemicals to look for, as these tests do not detect non-targeted contaminants or those with poor detection limits. With the exception of the use of theoretical bioaccumulation estimates for nonpolar organics, chemical analyses provide no information on the fraction of the targeted contaminant available (bioavailability) to cause toxicity and subsequent risks. Managers are then required to use lengthy bioaccumulation studies to assess contaminant exposure and bioavailability.

Confounding the remediation or mitigation of contaminated sediment, the causes of toxicity in sediment may never be conclusively identified using standard methods (Larsen 2001). As a result, the high cost of sediment analysis constrains effective management decisions. Given the large volumes of material involved in most dredging operations, the heterogeneous distribution of contaminants in sediments, and the frequent need to make decisions during dredging operations, the constraints imposed by traditional sampling regimes and analyses can result in overly conservative, and very expensive, decisions. Moreover, it may result in the unnecessary treatment of sediments where contaminants pose no risk. The lack of timely and accurate information can also result in localized hot spots escaping detection, and dispersal of toxic material into aquatic ecosystems. Direct assessment of effects precludes uncertainty due to unknown bioavailability improving the quality of information in terms of environmental risk. Introduction of rapid, inexpensive, and high-throughput screening methods, such as that described below, will significantly increase the amount

of material that can be assessed, resulting in more informed decision-making regarding the disposal of dredged material (Fredrickson et al. 2001).

INTRODUCTION: Sediment matrices create difficult challenges for identification of bioavailable contaminants that may cause toxicity. For a contaminant to cause any response, it must be biologically available. While bioavailability can seldom be determined from analytical chemistry data, responses in exposed organisms can be interpreted and used to identify which chemicals are bioavailable and their potential for causing effects and toxicity due to exposure.

When invertebrates are exposed to contaminants that are harmful, or biologically available at concentrations that may be harmful, a wide range of responses occurs based on the type of chemical and the level of exposure (Figure 1). The cells of an organism respond to stimuli by synthesizing proteins required for the cell to function (i.e., cell growth, cellular repair, and detoxification). Responses are created by transcribing, or copying many times, genes into messenger RNA (mRNA) that in turn are translated into proteins. At low levels of exposure, organisms generally attempt to eliminate or detoxify the contaminant. For example, mercury exposure results in accumulation of metallothionines to sequester mercury by increasing, or inducing, transcription of genes coding for the protein (Durnam and Palmiter 1981). Increasingly higher exposures cause cellular damage triggering compensatory pathways and repair mechanisms that mitigate the damage. Eventually, high levels of toxicants can cause organ damage and affect the animal's behavior (e.g., reproductive success and other common endpoints of chronic exposure bioassays). Exposures that overwhelm the cell's defense result in the transcription of a suite of genes that encode enzymes associated with cell death.

Chironomus tentans contains thousands of genes that provide it with many biochemical means to deal with low-level toxicant exposures. Therefore the amount of mRNA that accumulates for specific genes can be used to determine how much of a dose an animal receives. The bioavailability of a chemical will be indicated by changes in the types of mRNA that are produced in an animal as stress responses begin to occur. As a result the profile of genes transcribed is toxicant- and dosage-specific (Burczynski et al. 2000; Waring et al. 2001a, 2001b; Bartosiewicz et al. 2001; Gerhold et al. 2001).

mRNA PROFILING: To use mRNA profiling to identify the agent causing toxicity in an environmental sample, test organisms are exposed for a short period to the sediment to be analyzed and surviving animals are recovered. RNA is extracted from exposed organisms, providing a "snapshot" of the proteins the organism is currently synthesizing. A cell makes mRNA so that it can make more enzymes or other proteins; therefore, the types and levels of gene transcripts in the extract provide a snapshot of the cell's status (Figure 2).

Under controlled test conditions, toxicants having similar modes of action will have generally similar patterns of *C. tentans* gene expression. Experiments with rats and rat hepatocyte cell lines exposed to 15 known liver toxins demonstrate that mRNA profiles can be clustered into compounds with similar toxic mechanisms (Waring et al. 2001a, 2001b). However, the complete gene expression profile is unlikely to be the same for any two toxicants. That is, while rat liver toxicants

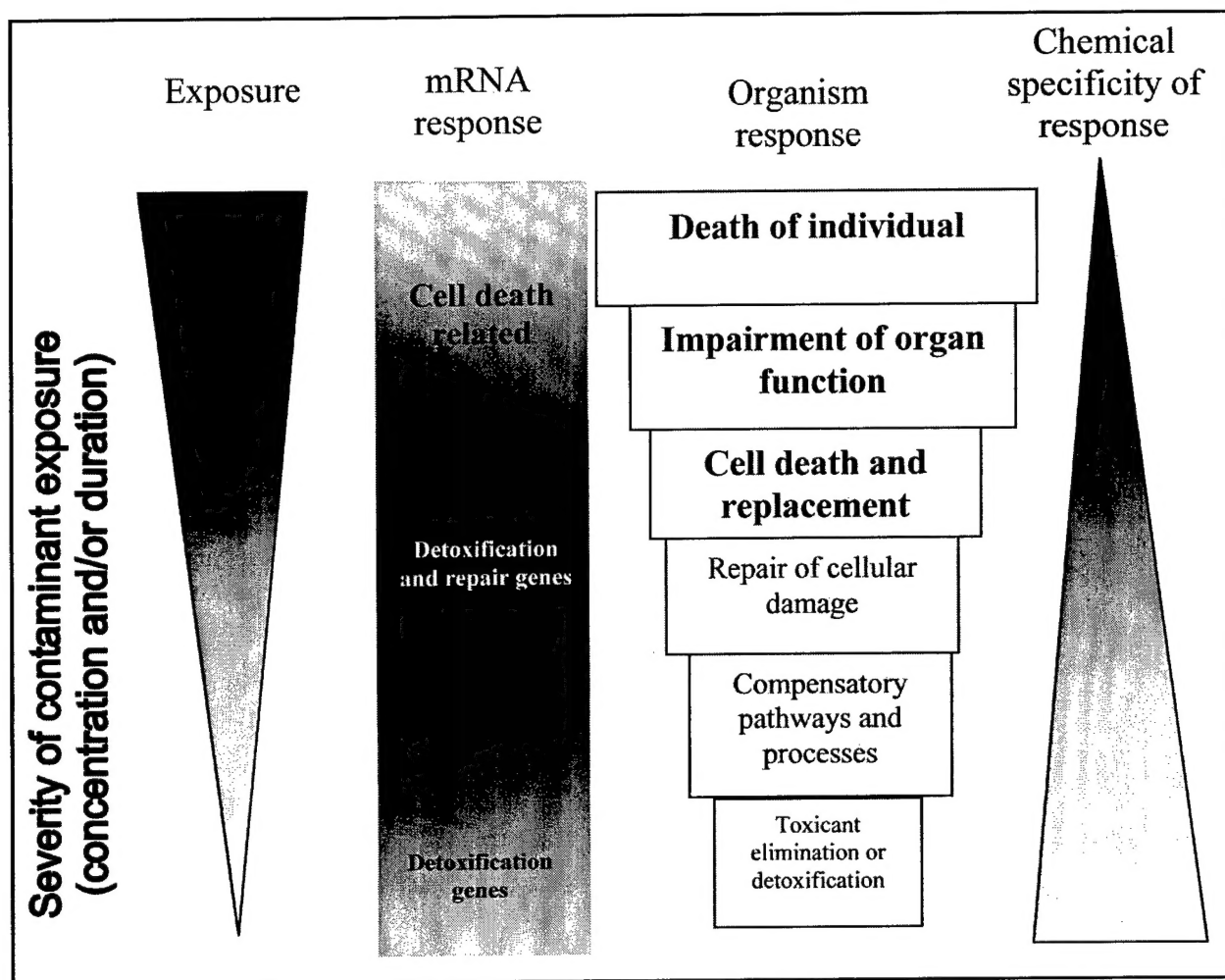


Figure 1. Relationships between exposure levels, mRNA profile, organism-level effects, and specificity of the responses with respect to the toxicant

with known and related modes of action produced generally similar expression profiles, each exposure resulted in a unique, reproducible gene expression profile. mRNA profile patterns in rats also correlated with cellular histopathology and biochemical changes in rat physiology directly linking changes in transcription profiles with whole organism toxicity.

Viewed from a different perspective, under controlled test conditions, gene expression profiles can be used to identify which contaminants *C. tentans* is sensing and, possibly, also the dosage of the toxicant. However, before this approach can be practically applied, a knowledge base of *C. tentans* mRNA profiles in response to specific toxicants must be compiled.

CHIRONOMUS TENTANS CONTAMINANT mRNA PROFILES: Although this approach can be used for any organisms and stressor, the authors chose to develop it for the transcriptional responses of *Chironomus tentans* to common sedimentary chemical contaminants. *C. tentans* can be used for both sediment and water testing, and there are standard EPA and ASTM protocols for its

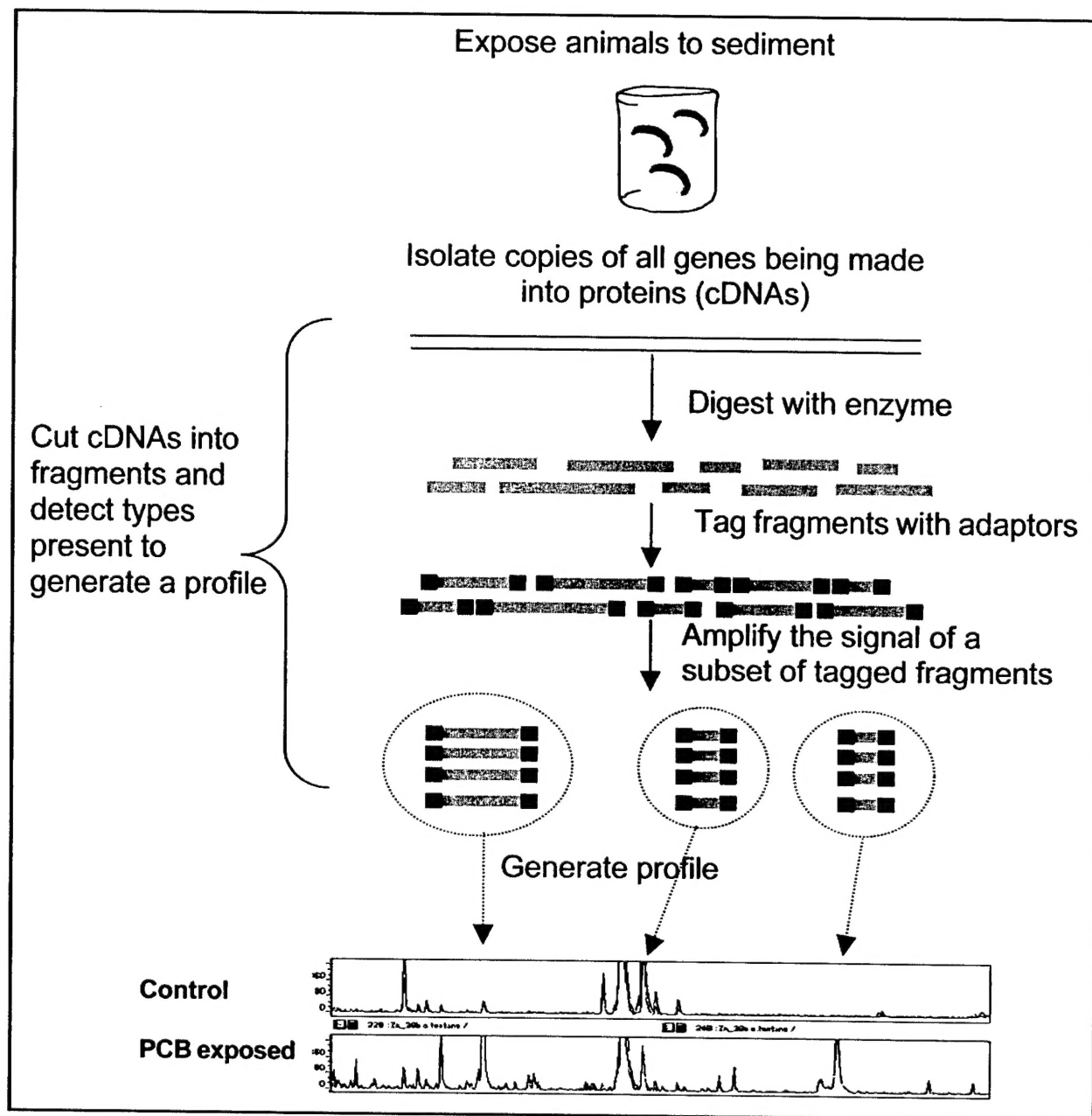


Figure 2. A profile of mRNAs is obtained by extracting and purifying mRNA from exposed and control *Chironomus tentan*. mRNA is reverse transcribed to cDNA, cut with restriction enzymes, and ligated to short pieces of DNA with known sequences (adaptors). Subsets of the adapted cDNA fragments are amplified using PCR. The resulting cDNA fragments are separated and visualized using an automated DNA sequencer. Each of the resulting peaks represents the expression of a single gene. These mRNA profiles are diagnostic for the type and severity of stress the cell was experiencing at the time of nucleic acid extraction

use in sediment testing (USEPA 2000). Chironomids are important prey for many fish including juvenile endangered salmon species. In studies of the diet of Chinook fry in the Sacramento River, CA, chironomids comprised 44 percent of the stomach contents of salmon fry, outnumbering any other item in the diet (Moore 1997).

This approach was demonstrated by mRNA profiling of *C. tentans* larvae exposed to six common sedimentary contaminants (Figure 3). Midge larvae were exposed for 12 hr to water containing dosages approximating one-tenth published LD₅₀'s; phenanthrene (25 µg/L) and fluoranthene (30 µg/L), DDT (0.2 µg/L), zinc chloride (125 µg/L), cupric chloride (54 µg/L), and cadmium chloride (100 µg/L). Total RNA was isolated from larvae in each exposure and analyzed using the method outlined in Figure 2. Different sets of adaptors can be selected so that the total population of mRNA-derived cDNA can be comprehensively interrogated by iteratively amplifying different subsets of the cDNA population. The adaptors are used to detect different cDNAs and hence different genes being expressed.

Each exposure produced a unique and reproducible gene expression profile. Toxicants with similar modes of action (e.g., phenanthrene and fluoranthene) produced expression profiles that were more similar to each other than to those of other toxicants (e.g., metals or DDT) (Figure 4).

CONCLUSIONS: Molecular-based biological toxicity assays can be used to greatly increase the quality, quantity, and timeliness of data required for rational management of dredged materials. mRNA responses of the sedimentary macrofaunal species *C. tentans* provide a direct biological assay for assessing bioavailability of contaminants in sediments and dredged materials. This profiling will also be useful in environmental forensics. By comparing of a contaminated sediment to potential source materials, mRNA profiles could be used to identify the source, and responsible party, of a contamination. Bioassays preclude concerns over missing hazardous sediments due to unexpected contaminants and the synergistic effects of chemical mixtures. Direct bioassays can help target remedial action resources to the bioavailable fraction of contaminated sediments. This capability is particularly useful when assessing hazards posed by heavy metals whose toxicity is a function of valence state and associated molecular structure. *C. tentans* gene mRNA profiles are diagnostic for the types and levels of contaminant exposure. Therefore, *a priori* knowledge of the contaminants present is not required for potential hazards to be identified. Genes can be induced by exposures of less than 1 hr and the entire analysis can be completed in 8 hr. Automated preparation and DNA sequences enable the analysis of hundreds of samples per day and even higher sample throughput is possible using high-density DNA microarrays. Current costs will rapidly decline as this technology transitions out of research and into commercial contract laboratories.

The authors will continue to develop this technology using *C. tentans* and the saltwater bioassay species *Leptochierus plumulosus*. Additional work is required in the design of exposures that control gene expression, and focus analytical methods to minimize the reporting of gene expression that is not related to toxicity. This streamlining will reduce both costs and potentially confounding results, and will assist in transitioning this technology to the private sector.

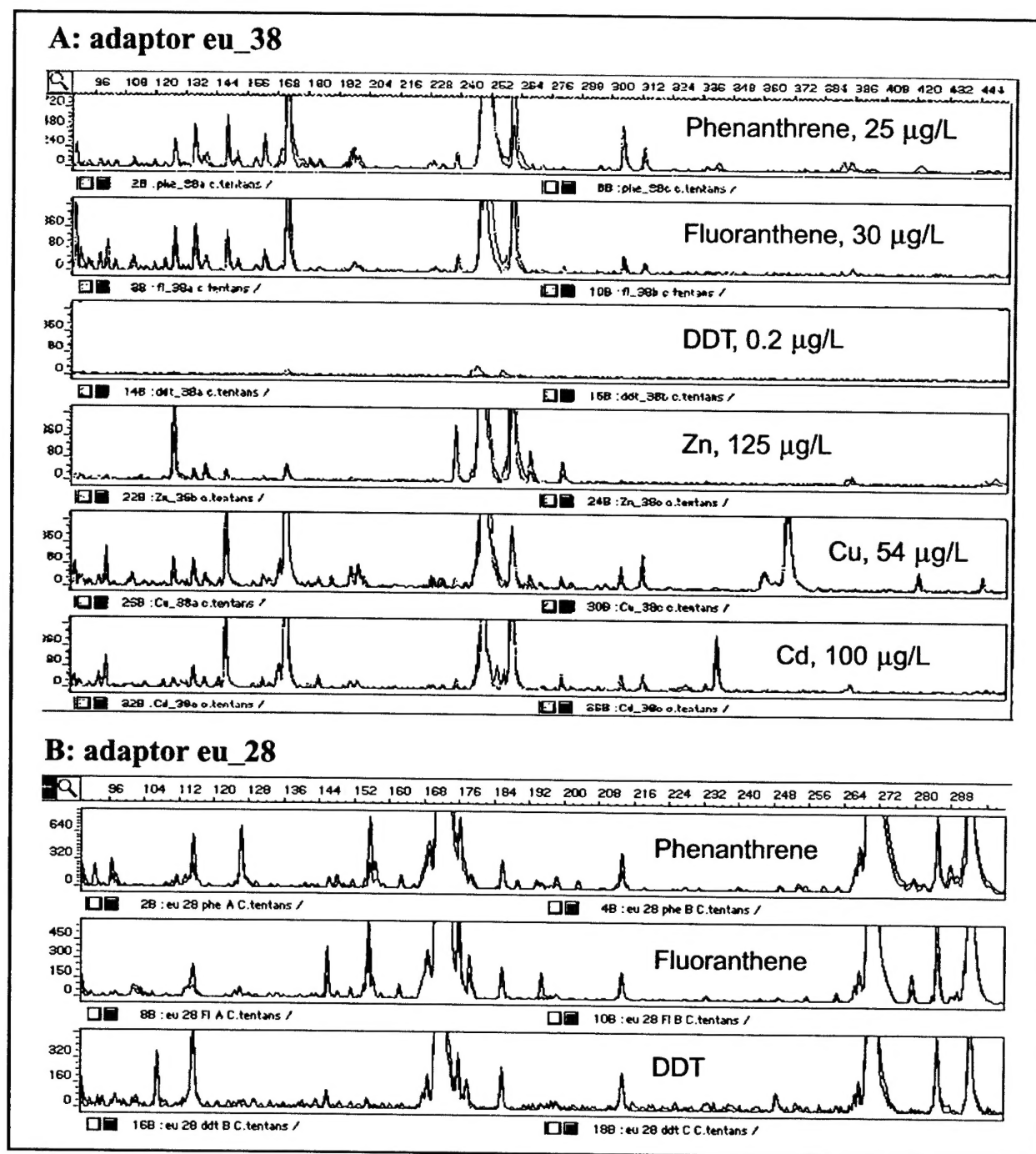


Figure 3. mRNA profiles of *C. tentans* exposed for 16 hours to sublethal levels of one of six different common sedimentary contaminants. Each panel contains the gene expression profiles from duplicate exposures and analyses. Total RNA extracted and purified from *C. tentans* exposed to the indicated contaminants was profiled using the method outlined in the previous figure. Each peak represents the expression of a single gene and the profile of peaks depicts part of the cellular response to the toxic insult. **A:** *C. tentans* toxic response mRNA profiles revealed using the adaptor eu_38. **B:** *C. tentans* toxic response mRNA profiles revealed using the adaptor eu_28. Note that while each expression profile is unique, toxicants with a probable similar mode of action express a more similar profile than those with different modes of action

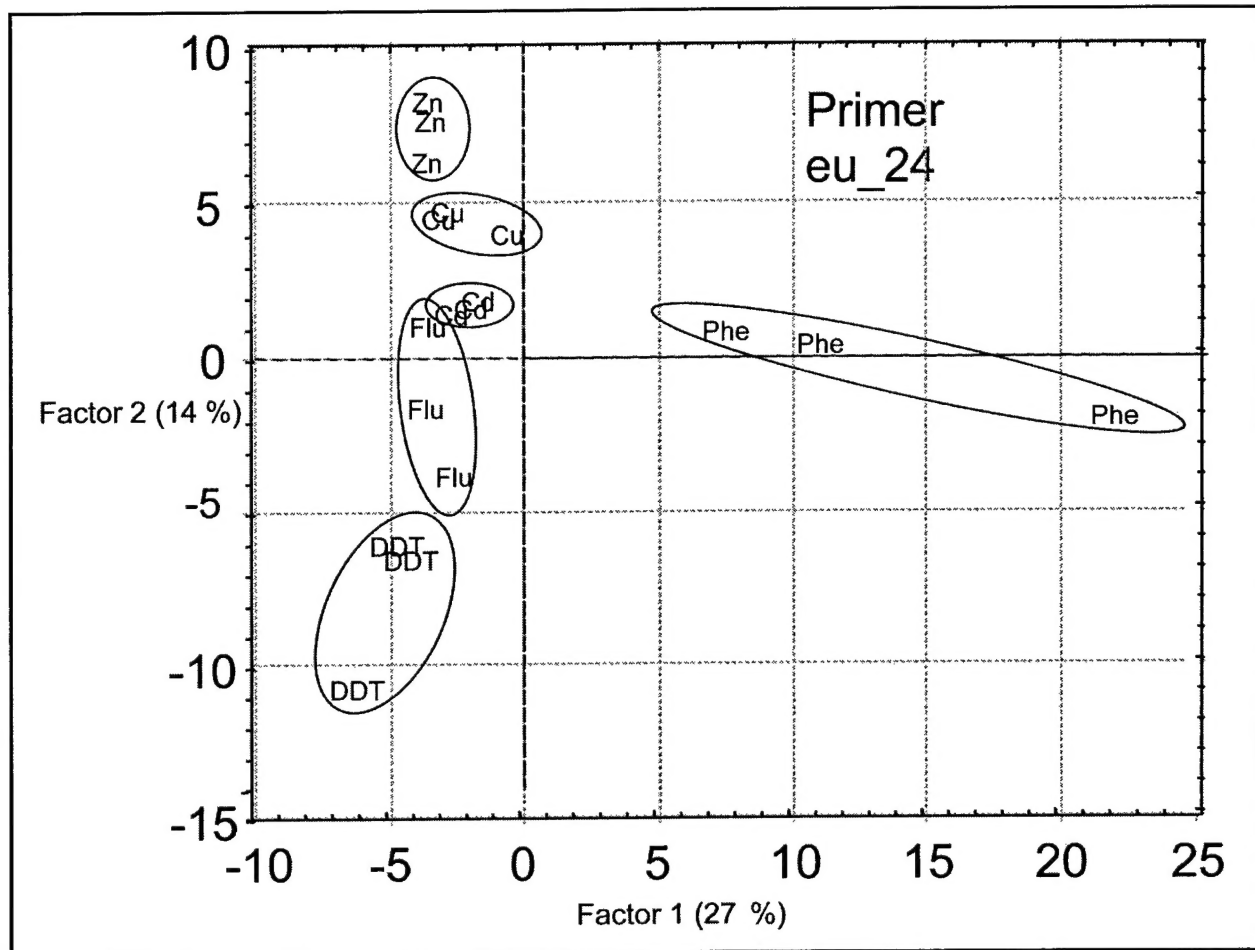


Figure 4. *C. tentans* mRNA profiles revealed using one adaptor (eu_24) were characterized by the gene fragment base pair length and normalized peak height of each peak. The resulting data were subjected to a Principle Component numerical analysis and the sample scores of each profile were plotted on the two factors that best differentiated the mRNA profiles. Points representing replicate mRNA profiles from animals exposed to contaminants formed clusters (Phe = phenanthrene exposed; Flu = fluoranthene exposed; DDT = DDT exposed; Cu = copper exposed; Cd = cadmium exposed; Zn = zinc exposed)

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REFERENCES

Bartosiewicz, M. J., Jenkins, D., Penn, S., Emery, J., and Buckpitt, A. (2001). "Unique gene expression patterns in liver and kidney associated with exposure to chemical toxicants," *J Pharmacol Exp Ther.* 297, 895-905.

- Burczynski, M. E., McMillian, M., Ciervo, J., Li, L., Parker, J. B., Dunn, R. T., Hicken, S., Farr, S., and Johnson, M. D. (2000). "Toxicogenomics-based discrimination of toxic mechanism in HepG2 human hepatoma cells," *Toxicol. Sci.* 58(2), 399-415.
- Durnam, D. M., and Palmiter, R. D. (1981). "Transcriptional regulation of the mouse metallothionein-I gene by heavy metals," *J Biol. Chem.* 256, 5712-6.
- Fredrickson, H. L., Perkins, E., Bridges, T. S., Tonnuci, R., Fleming, J., Nagel, A., Diedrich, K., Mendoza-Tenorio, A., Doltz, M. J., and Beattie, K., (2001). "Towards environmental toxicogenomics- development of a flow-through, high-density DNA array and its application to ecotoxicity assessment," *The Science of the Total Environment* 274, 137-149.
- Gerhold, D., Lu, M., Xu, J., Austin, C., Caskey, C. T., and Rushmore, T. (2001). "Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays," *Physiol Genomics* 27, 161-70.
- Larsen, K. (2001). "Strategy to address toxicity of unknown cause," Report of the Central Valley Regional Water Quality Control Board, Sacramento, CA. (Draft available: not yet finalized).
- Moore, T. L. (1997). "Condition and feeding of juvenile chinook salmon in selected intermittent tributaries of the upper Sacramento River," MS thesis, California State University, Chico, CA.
- U.S. Environmental Protection Agency. (2000). *Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates*. 2nd ed., EPA/600/R-99/064, Office of Research and Development and Office of Water, Washington, DC.
- U.S. Environmental Protection Agency/U.S. Army Corps of Engineers. (1991). *Evaluation of dredged material; proposed for ocean disposal (testing manual)*. EPA-503/8-91/001, USEPA Office of Marine and Estuarine Protection, Washington, DC.
- Waring, J. F., Ciurlionis, R., Jolly, R. A., Heindel, M., and Ulrich, R.G. (2001). "Microarray analysis of hepatotoxins in vitro reveals a correlation between gene expression profiles and mechanisms of toxicity," *Toxicol Lett.* 120, 359-68.
- Waring, J. F., Jolly, R. A., Ciurlionis, R., Lum, P. Y., Praestgaard, J. T., Morfitt, D. C., Buratto, B, Roberts, C., Schadt, E., and Ulrich, R. G. (2001). "Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles," *Toxicol Appl Pharmacol.* 175, 28-42.

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